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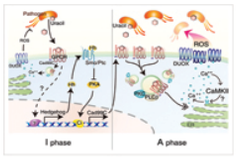

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Expanding the Iroquois genes repertoire: a non-transcriptional function in cell cycle progression

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***Drosophila* Iroquois (Iro) proteins are components of the TALE homeodomain family of transcriptional regulators. They play key roles in territorial specification and pattern formation. A recent study has disclosed a novel developmental function of the Iro proteins. In the eye and wing imaginal discs, they can regulate the size of the territories that they specify. They do so by cell–autonomously controlling cell cycle progression. Indeed, Iro proteins down-regulate the activity of the CyclinE/Cdk2 complex by a transcription-independent mechanism. This novel function is executed mainly through 2 evolutionarily conserved domains of the Iro proteins: the Cyclin Binding Domain and the IRO-box, which mediate their binding to CyclinE-containing protein complexes. Here we discuss the functional implications of the control of the cell cycle by Iro proteins for development and oncogenesis.**

Keywords: cell proliferation, *Drosophila*, imaginal discs, *Iroquois* genes, tumor suppressor genes

List of abbreviations and acronyms: *ara*, *araucan*; CBD, Cyclin Binding Domain; Cdk2, Cyclin-dependent kinase 2; *caup*, *caupolican*; HD, homeodomain; *Iro*, *Iroquois*; *Irx*, *Iroquois-related genes*; *mirr*, *mirror*; TALE, Three Amino acid Loop Extension; TSG, Tumor Suppressor Genes

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Introduction

It is more than 30 y since the discovery of the homeobox, an evolutionarily conserved DNA sequence found in the *Drosophila* homeotic genes.[1,2] The homeobox encodes a 60 amino acid-long domain that allows DNA binding. This domain was subsequently found in a plethora of transcription factors (103 in *Drosophila*), collectively known as homeoproteins. Like those encoded by the homeotic genes, homeoproteins are key players in embryonic and post-embryonic development (reviewed by 3, 4.) The homeodomain consists of 3 α helices surrounding a hydrophobic core.[3] The TALE subfamily of homeoproteins is characterized by the presence of a Three Amino acid Loop Extension between the first and second helix of the homeodomain.[5] Prominent members of this group are the products of the *Drosophila Iroquois* (*Iro*) complex genes, *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*), and their vertebrate orthologs, the Irx genes (reviewed by 6).

Iro genes play key roles during *Drosophila* development. At the early second larval instar, their expression in the eye precursor (the eye imaginal disc) defines the dorsal compartment of the eye.[7,8] Simultaneously, their expression in the mesothorax and wing precursor (the wing imaginal disc) defines the extent of the notum (dorsal mesothorax) territory.[9] Territorial specification by Iro proteins is not restricted to the imaginal discs. Thus, Mirr, through repression of *pipe*, defines the dorsal domain of the follicular epithelium of the egg chamber, which is required for the correct establishment of the embryonic axes.[10]

Iro proteins are also deeply involved in cell fate specification and pattern formation. For instance, they are components of the set of transcription factors that build up the pre-pattern that regulates expression of the proneural genes of the *achaete-scute* complex (AS-C) in proneural clusters ([11]; reviewed by 12.) Or, by controlling the expression of the *slouch* gene, Ara and Caup specify the lateral transverse muscle fate.[13]

Iro genes contribute to territorial growth in the eye and wing imaginal discs by generating organizing borders at the

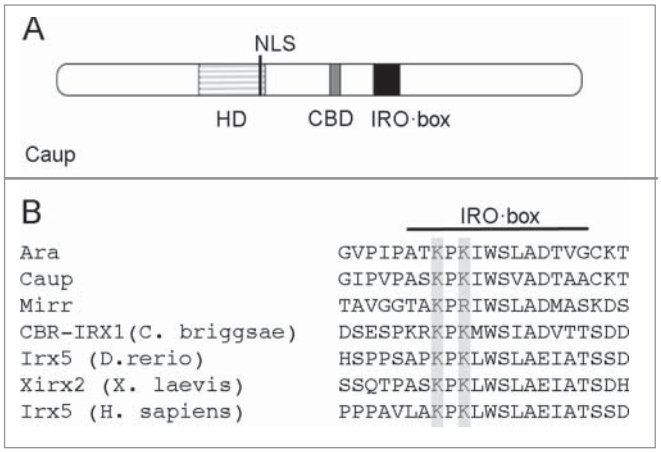

Figure 1. (**A**) **Domain structure of *Drosophila* Caup**. The evolutionary conserved homeodomain (HD); Cyclin biding domain (CBD) and the IRO·box are represented. NLS, Nuclear Localization Signal. (**B**) **Alignment of IRO·box sequences from different Iro and Irx proteins**. Note the strict conservation of the 2 lysine residues (marked in gray).

confrontation of *Iro*-expressing and non-expressing cells (the dorso/ventral and the notum/wing hinge organizers, respectively.[7-9,14]) In addition, the observation that clones of *iro* mutant cells in the eye disc are larger than wild-type clones suggested that Iro proteins played a role in the control of cell proliferation.[8,15] Indeed, Iro proteins cell-autonomously restrain cell proliferation in *Drosophila*, both during normal development and in several established tumor-like models.[16] More specifically, Iro proteins antagonize the activity of the Cyclin E (CycE)/Cyclin-dependent kinase 2 (Cdk2) complex, thus regulating the G1-S transition of the cell cycle. Unexpectedly, these Iro transcription factors do so by a non-transcriptional mechanism. The Iro/Irx proteins contain 2 evolutionarily conserved domains C-terminal to the homeobox, namely, a putative Cyclin-Binding Domain (CBD) and the IRO-box, hitherto of unknown function[5] (**Fig. 1**). Both domains appear to be involved in the physical interaction of Caup with CycE -containing protein complexes in S2 cells and in cell cycle regulation *in vivo*.[16]

Iro Proteins Cell-Autonomously Control Cell Cycle Progression

Flies harbouring combinations of hypomorphic *iro* alleles showed dorsal eye overgrowths.[16] Such overgrowths were not associated with the generation of ectopic dorso/ventral organisers. Interestingly, this phenotype was more frequent when *string* (*stg, Drosophila* ortholog of the cdc25 phosphatase that promotes the cell cycle transition from G2 to M[17]) was over-expressed in the eye discs of these mutants. This suggested a deregulation of the cell cycle. Indeed, quantification of the number of mitotic cells in the dorsal compartment of these discs (the realm of expression of the *Iro* genes) showed an increase of cell proliferation, as compared with that of wild-type discs. An increase was also observed in the *Iro* territory of the wing discs (the prospective notum). Importantly, the mitotic index in *Iro* non-expressing regions was unaffected in *iro* mutant discs, suggesting a cell-autonomous restriction of cell proliferation by the *Iro* genes. In agreement with this inference, over-expression of any of the *Iro* genes reduced cell proliferation in the disc's notum and wing territories.

Two findings strongly supported that the *Iro* genes act mainly at the G1/S transition of the cell cycle. First, cell cycle profile analysis of *iro* mutant cells showed a reduced fraction of cells in G1 and an increased fraction in S and G2 phases. This profile is very similar to that found in wing disc cells over-expressing *CycE*.[18] Second, co-expression of *Iro* genes with *CycE*, but not with *CycA* or *stg*, normalized the proliferation of *Iro* over-expressing cells.

The IRO-box as an Important Domain for the Regulation of Cell Cycle Progression

In addition to the homeodomain, the Iro/Irx transcription factors share 2 other conserved domains, namely, the IRO-box,[5] whose function had not yet been established, and a putative Cyclin Binding Domain (CBD, **Fig. 1A**). Several recent data have assessed the relevance of these 3 domains for cell cycle control.

In the case of the Caup homeodomain, Asparagine 51 (N51) or Arginine 55 (R55) and Arginine 57 (R57) of the DNA recognition helix of the homeodomain were mutated to Alanine (*caup*^{HD*} mutants). Other work had shown the importance of these amino acids for DNA recognition in other homeoproteins.[19,20] We observed that the Caup^{HD*} mutant proteins were located in both the nucleus and in the cytoplasm of wing discs cells. This identified amino acids N51, R55 and R57 of the Caup homeodomain as part of a Nuclear Localization Signal (NLS, **Fig. 1A**), in agreement with the localization of NLS in other homeoproteins at the N-terminus of the homeodomain.[21,22] While, as expected, wild-type Iro proteins repressed *fng* transcription in the eye disc,[23-25] the mutant Caup^{HD*} proteins did not do so. However, they retained their capability to slow-down cell cycle progression when overexpressed. These observations indicated that the ability of Caup to repress transcription and to slow-down cell cycle are 2 separable functions, likely executed by different protein domains.

The Caup IRO-box, a highly conserved 14 amino acid-long domain (**Fig. 1B**), was mutagenized at its 2 positively charged amino acids, while the CDB was deprived of 3 out of the 5 amino acids of the predicted domain. Both of these modified Caup proteins still inhibited *fng* expression in the eye disc, but had a strongly reduced ability to inhibit cell proliferation. This suggested that both domains collaborate to arrest cell cycle. Since these modifications at the IRO-Box and CBD did not interfere with the transcriptional activity of Caup, we proposed that cell cycle regulation by Caup (and by extension, by Ara and Mirr

since they also contain IRO-Box and CBD, **Figure 1B** and not shown) does not depend on their well-known function as transcription factors. These experiments disclosed, for the first time, a non-transcriptional function of the Iro proteins.

The Level of Iro Proteins Sets a Functional Threshold for the Activity of the CycE/Cdk2 Complex

Mechanistically, it was shown that the overexpression of *caup* in the wing disc decreased the activity of the CycE/ Cdk2 complex,[16] the main regulator of the G1/S transition in *Drosophila*.[26] Activity of this complex is regulated by the cycling levels of CycE and by interaction with CDK inhibitors such as Dacapo (Dap), the ortholog of vertebrate p21 (**Figs. 2A, B**). However, in cells over-expressing *caup*, neither the mRNA nor the protein levels of *dap* were affected. Transcription of *CycE* was similarly unaffected. Unexpectedly, the *caup* over-expressing cells accumulated high levels of CycE protein. (This enhanced accumulation of CycE probably resulted form impaired activity of the CycE/Cdk2 complex, since Cdk2-dependent phosphorylation of CycE is a prerequisite for its degradation through the proteasome pathway.[27]) In spite of this accumulation, CycE appeared to be a limiting factor in cell proliferation, since its exogenous administration restored normal levels of cell cycling to the *caup* over-expressing cells. This unexpected observation might be explained by the finding that Caup can bind to CycE-containing protein complexes and restrict the activity of the CycE/Cdk2 complexes (**Fig. 2B**). Interestingly, the IRO-box and CBD Caup mutant proteins, which were largely impaired in arresting the cell cycle *in vivo*, also had a highly reduced ability to co-immunoprecipitate with CycE. Accordingly, we proposed that Caup physically interacts with CycE-containing complexes, by means of the IRO·box and CBD domains, and inhibits CycE/Cdk2 complex activity. Further work is required for a more precise definition of the molecular mechanisms underlying such functional inhibition. Thus, direct interaction of Caup with CycE and/or Cdk2 has yet to be investigated. In addition, presently, it cannot be discerned whether the interaction of Caup with CycE-containing complexes destabilizes them or whether it inhibits their activity by stabilizing Dap binding and/or preventing substrate recognition.

Considering that all Iro proteins contain the IRO-box (**Fig. 1B**) and the

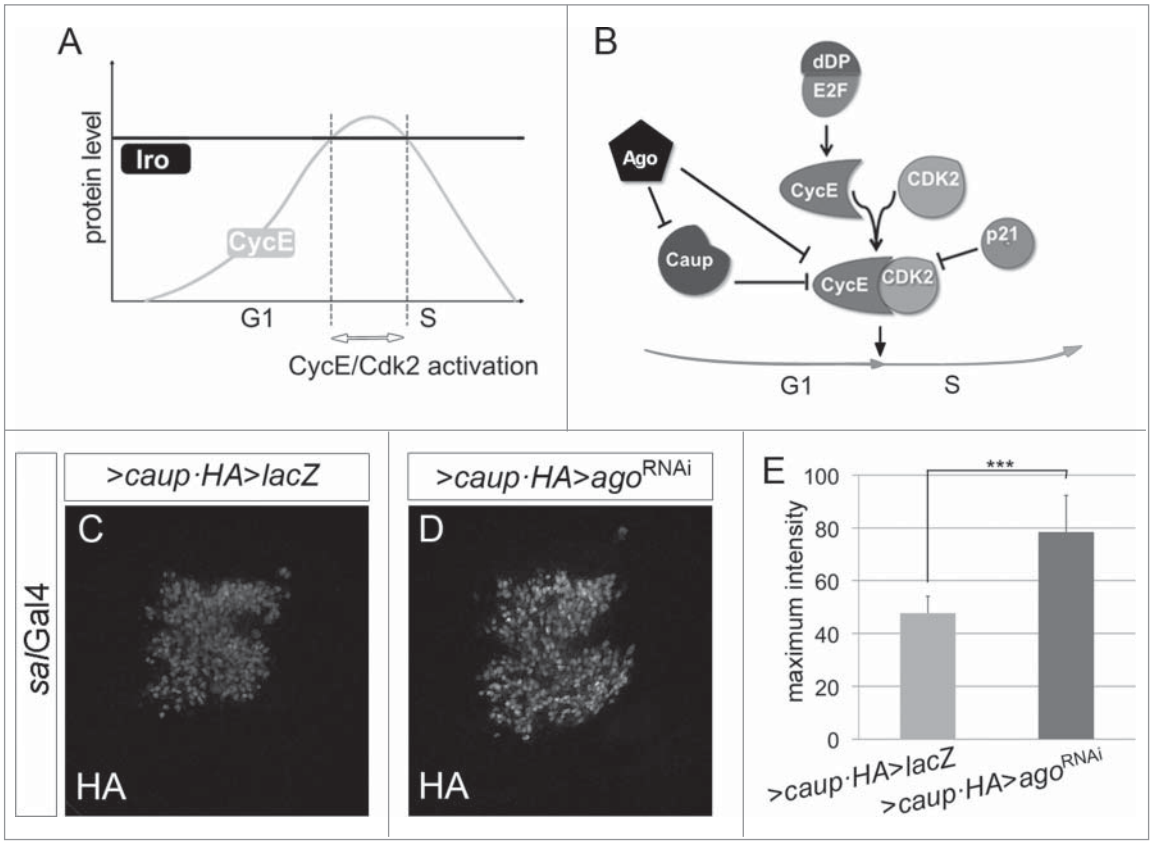

Figure 2. (A, B) Model for the regulation of cell cycle progression by Iro proteins. (A) The level of Iro proteins sets a threshold for CycE/Cdk2 activity. The G1/S transition does not occur until CycE accumulates above that threshold. (B) Caup controls the G1/S transition by binding to CycE-containing complexes and restricting the activity of the CycE/Cdk2 complexes. (C-E) Post-transcriptional control of Caup. Accumulation of exogenously provided Caup-HA (*sal*Gal4 driver) in control (C) or Ago-depleted (D) wing disc cells. (E) Quantification of the maximum pixel intensity of HA staining in cells that over-express the indicated transgenes (n = 6).

CBD, and all of them arrest the cell cycle when over-expressed, it is likely that they all can bind to CycE containing complexes. This hypothesis would explain the observation that the lower the doses of *Iro* genes, the more frequently the dorsal eye overgrowths. Thus, we propose that the amount of Iro proteins in the cell sets a threshold for CycE/Cdk2 activity such that the G1 to S transition would not take place until the accumulated level of CycE ensured triggering of the process (**Fig. 2A**).

A corollary of this model is that proper cell cycle progression requires a tight regulation of Iro levels. In addition to the previously described transcriptional mechanisms that regulate the expression of the Iro genes,[7,14,28-36] we now propose that there should also exist post-transcriptional mechanisms to ensure an efficient control of the levels of Iro proteins. In this context, it is of interest that we have indications that depletion of the F-box protein Archipelago (Ago), which induces the degradation through the proteasome pathway of the cell proliferation-promoting proteins CycE and Myc,[37,38] stabilizes exogenously provided Caup (**Figs. 2C-E**).

Integrating Cell Cycle Control by Iro Proteins in Current Models for Territorial Specification

Iro genes play a direct cell-autonomous role in territorial specification in the imaginal discs.[7,9] Now, we have shown their ability to, in addition, control the size of the territories they specify.[16] We consider this of interest in the context of current models for territorial specification. Thus, in the wing disc, wing specification driven by Wingless (Wg) is counteracted by Vein (Vn), which spreads from the most proximal part of the wing disc (**Fig. 3**, left top). Wing differentiation is precluded until the disc reaches a critical size that allows Wg to escape repression from its inhibitor Vn (**Fig. 3**, left bottom, reviewed by 39.) Since the Iro genes are able to restrain the size of the notum domain, we propose that they may facilitate the antagonistic action of Vn on Wg by delaying the separation of the Vn source from its target cells of the putative wing domain. Hence, the Iro genes would participate in the proper control of the size of the wing discs to allow the timely specification of the wing. This model agrees with our observation that reduction of the size of the prospective wing pouch by ectopic expression of *ara* prevents wing development, and that this development is restored by CycE co-expression.[16]

An analogous scenario would operate in the eye disc. There, the ability of Decapentaplegic (Dpp) to induce retina differentiation is counteracted by Wg emanating from the anterior-most region of the discs (**Fig. 3**, right top, reviewed by 40.) Retina differentiation starts when the increase in disc size frees Dpp form Wg repression[41] (**Fig. 3**, right bottom). In *iro* mutant eye discs we found enhanced cell proliferation in the dorsal territory ahead of the morphogenetic furrow. This would enlarge the physical separation between the Wg and Dpp sources, thus increasing the efficiency of Dpp signaling and leading to the observed enlargement of the dorsal eye. Interestingly, Vein activates *Iro* gene expression in the wing disc, while Wg does so in the dorsal eye disc.[7,31-36] Thus, we further propose that *Iro* genes may provide a molecular mechanism by which the morphogens Vein (in the wing disc) and Wg (in the eye disc) regulate the size of the morphogenetic field where they spread and operate.

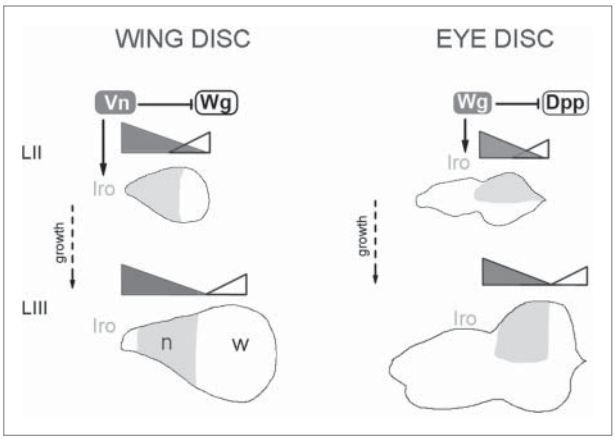

Figure 3. Control of cell proliferation by Iro-(C)genes in the imaginal discs and its involvement in territorial specification. (Left) In the wing disc, specification of the wing territory (w) by Wingless (Wg), counteracted by Vein (Vn), depends on the correct growth of the disc. Vn activates Iro expression in the notum territory (n, gray area). (Right) In the eye discs, Wg prevents specification of the eye by Dpp. Iro expression in the dorsal region of the eye disc (gray area) is regulated by Wg. Arrows and T-shaped bars respectively indicate positive and negative regulatory interactions. The dark gray and white triangles represent the level of activity of Vn and Wg (wing disc) or Wg and Dpp (eye disc). LII, LIII, second and third larval instars, respectively.

Inhibition of CycE/cdk2 Activity by Iro Proteins, a Mechanism for Tumor Suppression

Cell cycle deregulation lies at the heart of cancer. Indeed, the strict control of the activity of the CycE/Cdk2 complexes is often lost in tumor cells.[42] We have shown that Iro proteins counteract the overgrowth of imaginal discs in 2 established *Drosophila* tumor models.[16] Furthermore, in the *yorkie* over-expression model (*yorkie* is a core component of the evolutionarily conserved Hippo pathway that controls cell proliferation and apoptosis, reviewed by 43), the effect of *caup* is at least partially mediated by CycE/Cdk2 inactivation.[16] These data suggest that *Iro* genes may play a tumor suppressor role in *Drosophila*. Moreover, loss or reduced expression of members of *Irx* gene family are associated with several types of human cancer[44-47] and, accordingly, they are considered Tumor Suppressor genes (TSGs). The presence of IRO-box and CBD in Irx proteins allows us to hypothesize that Irx proteins may act as TSG by restricting cell

proliferation through a mechanism similar to that of *Drosophila* Caup, namely, interference with the G1-S transition by physical interaction with and inhibition of CycE-containing protein complexes.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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